

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12447

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/20, 38/16, 39/40, 39/095, 39/102, 39/395; C07K 1/00, 16/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/130.1, 150.1, 157.1, 164.1, 165.1, 169.1, 249.1, 256.1, 535; 426/580; 514/6; 530/350, 387.1, 387.15, 3 88.4, 389.1, 389.5, 400, 832

B. FIELDS SEARCHED

Minimum documentation searched
Classification System: U.S.

424/130.1, 150.1, 157.1, 164.1, 165.1, 169.1, 249.1, 256.1, 535; 426/580; 514/6; 530/350, 387.1, 387.15, 3 88.4, 389.1, 389.5, 400, 832

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PCT/US96/12447

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WITTLER et al. Neutralization of Streptococcus-Sanguis IgA1 protease by lactoferrin and transferrin. J. of Dental Research. 10 March 1993, Vol. 72, page 327, see entire document.	1, 6-11, 13-15, 18-19

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12447

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, BIOSIS, EMBASE, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y, P	LOMHOLT et al. Distinct antigenic and genetic properties of the immunoglobulin A1 protease produced by Haemophilus influenzae biogroup aegyptius associated with Brazilian purpuric fever in Brazil. Infection and Immunity. 01 November 1995, Vol 63, No. 11, pages 4389-4394, see entire document.	1-2, 8, 11, 14
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Y	MORELLI et al. Immunogenicity and evolutionary variability of epitopes within IgA1 protease from serogroup A Neisseria meningitidis. Molecular Microbiology. 01 January 1994, Vol. 11, No. 1, pages 175-187, see entire document.	1-2, 8, 11, 14
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Y	WO 93/10818 A1 (NEW ENGLAND MEDICAL CENTER HOSPITALS, INC) 10 June 1993, see entire document.	1-5, 8, 11-12
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☒ X

Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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19 SEPTEMBER 1996

31 OCT 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

Fig. 10B

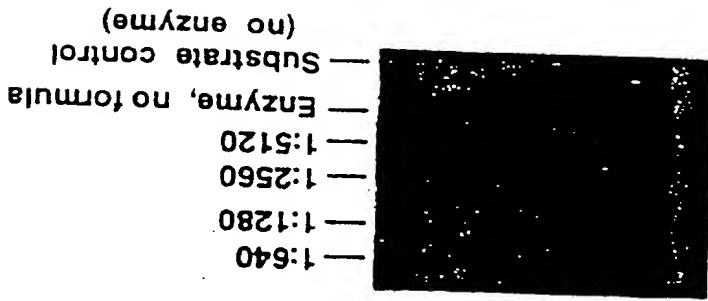
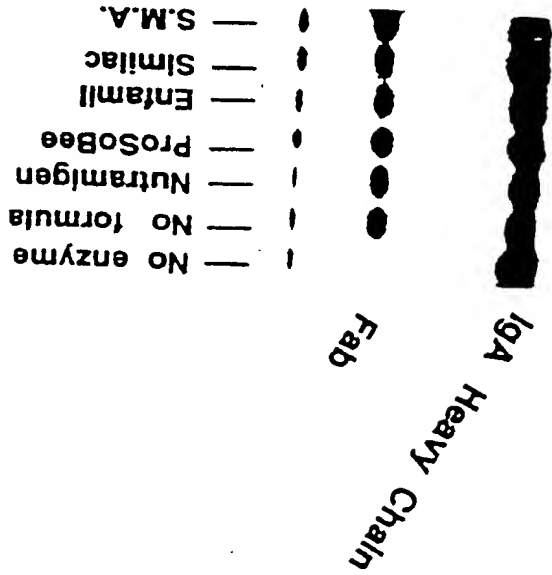


Fig. 10A



10/11

FIG. 9

IgA1 protease-inhibition activity^a (ELISA) of cow serum, colostrum and milk following immunization^b prior to a second lactation.^c

Cow #	Immunizing ^d	Serum				Colostrum		Milk ^f
		Day 0 ^e	Day 14	Day 21	Day 28	Day 35	Day 42	
339	<i>H. influenzae</i>	10	20	20	320	320	320	10
265	<i>N. meningitidis</i> 1C	40	80	80	640	640	640	10
342	<i>N. meningitidis</i> 1C	40	40	320	320	320	320	10
337	<i>N. meningitidis</i> 2R	40	80	320	1280	1280	1280	20
292	<i>S. sanguis</i>	0	0	0	0	0	0	0
301	<i>S. sanguis</i>	0	0	0	0	0	0	0
293	<i>S. pneumoniae</i>	0	0	0	0	0	0	0
324	<i>S. pneumoniae</i>	0	0	0	0	0	0	0

^a reciprocal of the last dilution in which optical density is two standard deviations above the negative control
^b cows were immunized on days 0, and 14 prior to expected calving date
^c refers to the second lactation in which these cows were observed in this study, but does not necessarily reflect actual cow parity
^d antigens were semi-purified IgA1-cleaving proteases of each bacterium listed
^e relative to expected calving date (day 28)
^f milk sample from one week postpartum

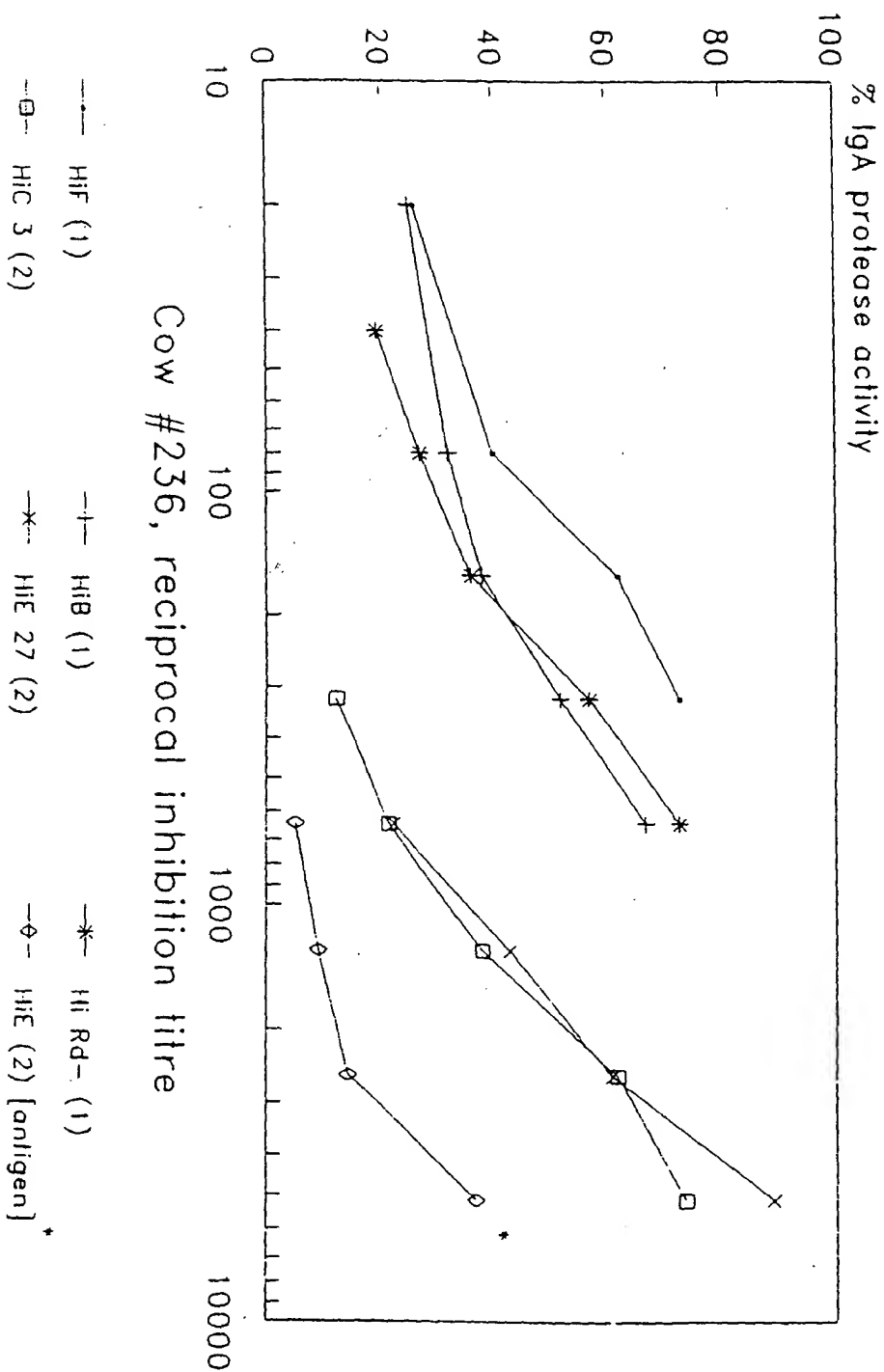


FIG. 88

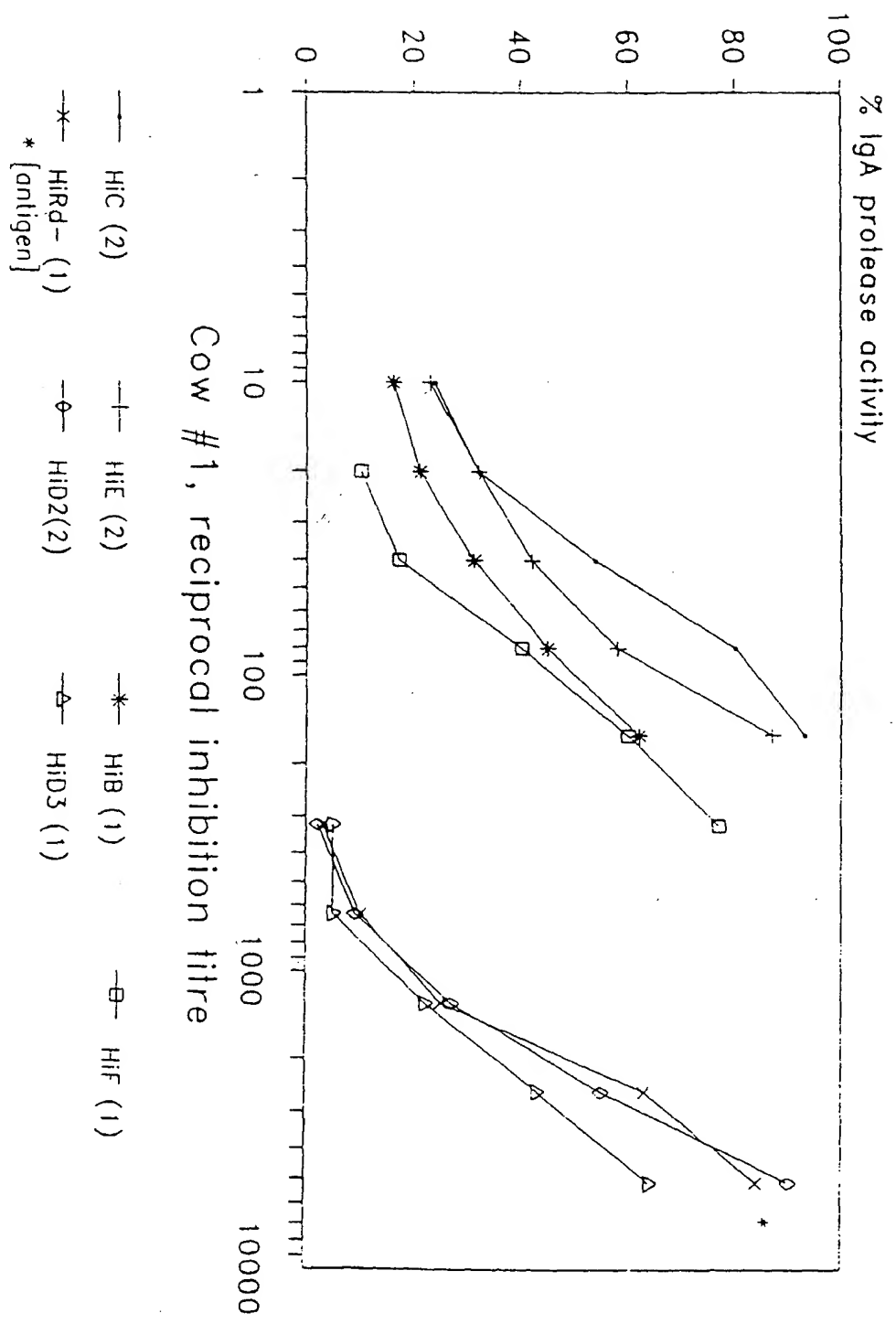


FIG. 8A

FIG. 7

IgA1 protease-inhibition activity^a (ELISA) of cow serum, colostrum and milk following immunization^b prior to a first lactation.^c

Cow #	Immunizing ^d	Serum				Colostrum		Milk ^e
		Day 0 ^e	Day 14	Day 21				
193	<i>H. influenzae e</i>	0	10	40	160	0		0
339	<i>H. influenzae e</i>	0	0	40	160	0		0
305	<i>H. influenzae b</i>	0	0	0	0	0		0
338	<i>H. influenzae b</i>	0	0	10	10	0		0
265	<i>N. meningitidis 1C</i>	0	40	80	40	0		0
342	<i>N. meningitidis 1C</i>	0	160	40	0	0		0
286	<i>N. meningitidis 2R</i>	0	20	40	0	0		0
337	<i>N. meningitidis 2R</i>	0	10	20	0	0		0
292	<i>S. sanguis</i>	0	0	0	0	0		0
301	<i>S. sanguis</i>	0	0	0	0	0		0
293	<i>S. pneumoniae</i>	0	0	0	0	0		0
324	<i>S. pneumoniae</i>	0	0	0	0	0		0

^a reciprocal of the last dilution in which optical density is two standard deviations above the negative control
^b cows were immunized on days 0, and 14 prior to expected calving date
^c refers to the first lactation in which these cows were observed in this study, but does not necessarily reflect actual cow parity
^d antigens were semi-purified IgA1-cleaving proteases of each bacterium listed
^e relative to expected calving date (day 28)
^f milk sample from one week postpartum

BOVINE ANTIBODY AGAINST IMMUNIZING PROTEASE

FOLLOWING SEQUENTIAL CALVINGS

(Reciprocal of IC_{50} titre)

Cow	Colost. calf #1	Colost. calf #2	Milk* (1 wk)
265	90	13,500	145
342	0	8,800	100
337	6	54,000	360
339	1,550	12,400	36
293	2	55	0
324	0	0	0
292	0	6	0
301	0	0	0

Antigens used...
265 *N. meningitidis* type 1
342 *N. meningitidis* type 1
337 *N. meningitidis* type 2
339 *H. influenzae* type 2
293 *S. pneumoniae*
324 *S. pneumoniae*
292 *S. sanguis*
301 *S. sanguis*

* Milk data are from second calving.

FIG. 6

BOVINE COLOSTRAL INHIBITION OF IgA PROTEASES FOLLOWING FIRST IMMUNIZATION

COW	N. men (1) *	N. men (2)	H. inf b (1)	H. inf e (2)	S. sanguis	S. pneum	H. inf Rd (1)	N. gon (2)
#272	295	42	28	17	0	0		
334	132	23	0	0	0	0		
265	90							
342	0							
286		3						
337		6						
305			5					
338			144					
1679			0	0	0		0	0
339	19	6	21	1550	0	0		
191				740				
236	23		136	3180	0	0	118	0
292					0			
301					0			
186			0		0			
293						2		
324						0		
194			46	27	0	0	910	0

* Number in parenthesis refers to enzyme cleavage type

FIG. 5

Fig. 4C
signal
depleted
milk
supplemented
with signal

Fig. 4B
signal
depleted
milk

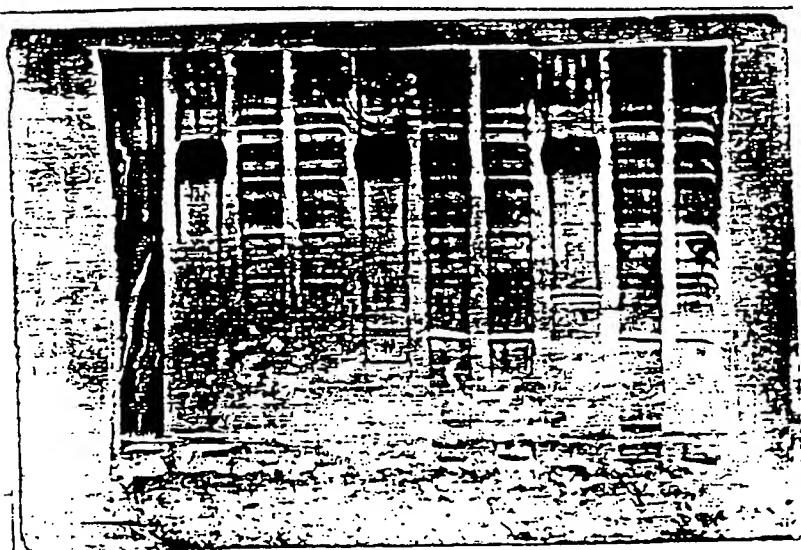
Milk

Fig. 4A

1 2 3 4 5 6 7 8 9 10

1. Culture supernatant of HI RD in BHI
2. Rd cell in BHI
3. Rd cell after growth in milk, signal depleted milk, or signal depleted with signal supplemented with signal
4. Milk sup. 0 min
5. Milk sup. 45 min
6. Milk sup. 90 min
7. Milk sup. 180 min
8. Milk sup. 9 hrs.
9. Milk sup. 24 hrs.
10. Milk only.

1. cells after 5 hrs. growth in BHI
2. cells after 5 hrs. growth in BHI, followed by 1 hr. growth in recombinant lactoferrin
3. recombinant lactoferrin supernatant after 1 hr. cell growth



1	2	3
HI Rd ⁻	HI mutant (3-13)	HI 1ga ⁻ (2-25DK)
size markers		

FIG. 3

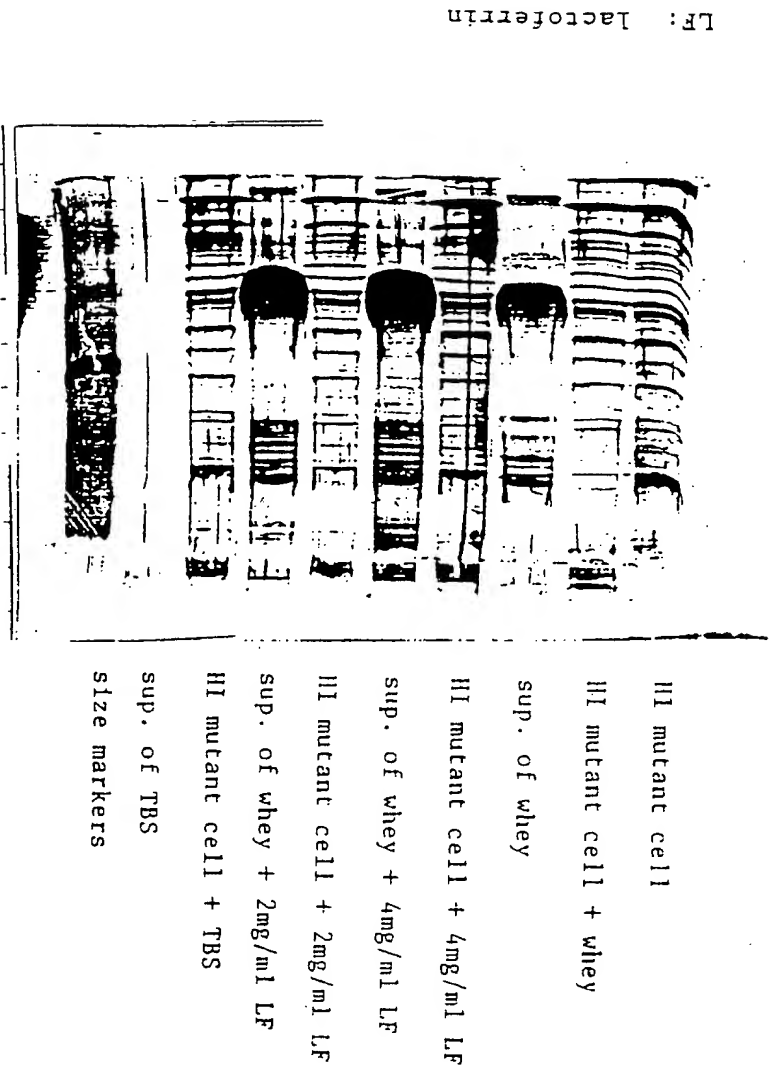


FIG. 2

HI mutant cell pellet

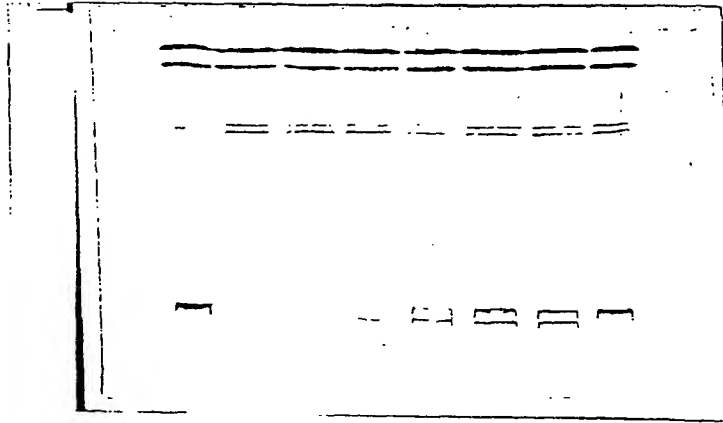


Fig. 1B

HI mutant cell, control
2 min whey + HI
5 min whey + HI
10 min whey + HI
20 min whey + HI
40 min whey + HI
80 min whey + HI
80 min saline sup. + HI

whey supernatant

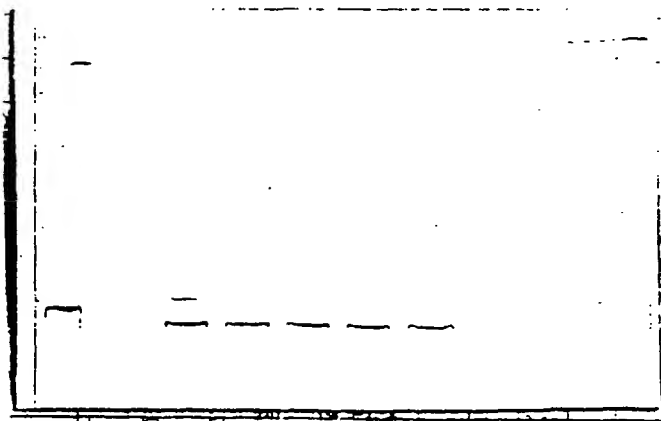


Fig. 1A

whey - HI
2 min whey + HI
5 min whey + HI
10 min whey + HI
20 min whey + HI
40 min whey + HI
80 min whey + HI
80 min saline sup. + HI
HI mutant cell, control

- contacting said extracted Iga protease precursor with an antibody which specifically binds said Iga protease.
15. A method of claim 14, wherein said 5 lactoferrin is non-pasteurized lactoferrin.
16. An antibody that specifically binds an Iga protease precursor and that does not specifically bind an Iga protease.
17. The antibody of claim 16, wherein said 10 antibody specifically binds the helper section of said Iga protease precursor.
18. A pharmaceutical formulation comprising active lactoferrin and an antibody which specifically binds either an Iga protease-like protein, or an Iga 15 protease precursor-like protein, or both an Iga protease-like protein and an Iga protease precursor-like protein.
19. A formulation of claim 18, wherein said lactoferrin is non-pasteurized lactoferrin.
20. The formulation of claim 18, wherein said 20 antibody specifically binds an Iga protease-like protein selected from the group consisting of vacuolating cytotoxin, Tsh protein, and HAP protein.

adding active lactoferrin to said milk to prepare said infant formula.

9. A method of claim 8, wherein said lactoferrin is human lactoferrin.

10. A method of claim 8, wherein said lactoferrin is non-pasteurized lactoferrin.

11. A method for producing an infant formula additive, said method comprising immunizing a pregnant non-human mammal with all or a portion of an IgA protease or an IgA protease precursor;

collecting from said mammal milk comprising antibodies which specifically bind either an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor;

pasteurizing said milk; and

mixing pasteurized milk with active lactoferrin to produce the infant formula additive.

12. A method of claim 11, wherein, prior to said mixing, fat and casein are removed from said milk.

13. A method of claim 11, wherein said lactoferrin is non-pasteurized lactoferrin.

14. A method for inhibiting proteolysis of an IgA1 antibody by an IgA protease, said method comprising contacting a cell comprising a precursor of said IgA protease with a formulation comprising a pasteurized excipient, and active lactoferrin, thereby producing an extracted IgA protease precursor; and

1. An infant formula comprising pasteurized milk, active lactoferrin, and an antibody that specifically binds either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor.
2. A formula of claim 1, wherein said antibody specifically binds an IgA protease.
3. An infant formula of claim 1, wherein said milk is non-human colostrum.
4. A formula of claim 3, wherein said colostrum is bovine colostrum.
5. A formula of claim 1, wherein said antibody is bovine IgG.
6. A formula of claim 1, wherein said lactoferrin is human lactoferrin.
7. A formula of claim 1, wherein said lactoferrin is non-pasteurized lactoferrin.
8. A method for preparing a pasteurized infant formula, said method comprising immunizing a non-human mammal with all or a portion of an IgA protease or an IgA protease precursor; collecting from said mammal milk comprising antibodies that specifically bind either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor; and pasteurizing said milk; and

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initiator of plaque formation which causes dental caries in adults. Lactoferrin and inhibiting antibodies can thus be formulated into a mouthwash or beverage, for example, for use in adults. The formulations can also be used in treating *Pneumococcus* infections in adults and children. These bacteria produce IgA proteases and are major lung pathogens, causing pneumonia in adults. Accordingly, the invention can be formulated for intrabronchial or intranasal administration. In addition, *Haemophilus influenza* also infects adults, and the invention thus can be used to inhibit HI IgA proteases in adults as well.

What is claimed is:

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

For example, the invention includes formulations that are made without milk, but which include lactoferrin and antibodies that inhibit the proteolytic activities of IgA proteases, protease precursors, protease-like proteins, and protease precursor-like proteins. Non-milk based formulations that include antibodies and lactoferrin can be used as infant formulas. Milk or non-milk formulations of the invention can be used to inhibit infections in adults. For example, *S. sanguis* is a major

the ratio of colostrum:formula (vol:vol) is 1:640, 1:1280, 1:2560, and 1:5120. The enzyme added to all these dilutions is inactive, as shown by the inability to fragment any ¹²⁵I-IgA substrate during 30 minutes.

In general, the bovine antibodies of the invention should be fed to an infant in combination with lactoferrin, preferably as a formula supplement, at a dosage of about 1.0 to 2.0 grams of bovine colostrum antibody protein/kg/day and 0.01 mg/ml to 10 mg/ml of lactoferrin/kg/day, preferably 0.5-1.0 mg/ml, equally distributed over all feedings. This dosage must be adjusted dependent on the titer of the antibodies in the particular colostrum preparation used as the infant formula additive, because different colostrum preparations have different percentages of the IgA protease antibodies compared to total antibodies in the preparation.

Other Embodiments

colostrum titers described above. The results showed that the formulas supplemented with these antibodies had essentially the same inhibitory effect as the colostrum whey preparation described above.

5 Figs. 10A and 10B are autoradiographs of polyacrylamide electrophoresis gels of ¹²⁵I-IgA1 substrate subjected to IgA protease. These autoradiographs show uncleaved (IgA heavy chain) substrate, and fragments arising from proteolysis (Fab). In all cases the protease assay was carried out for 30 minutes.

Fig. 10A shows that IgA protease is active when added to five commercial infant formulas, whereas Fig. 10B shows that the IgA protease is inactive when added to formula supplemented with the inhibiting bovine antibodies of the invention. The colostrum of cows never immunized with proteases, which lacks antibody, was used as a control.

Fig. 10A shows the gel resulting from *Haemophilus influenzae* type 1 IgA protease added to a number of commercial infant formulas, along with trace amounts of ¹²⁵I-IgA substrate. The top two lanes are controls: the top lane is substrate without protease, which resulted in no cleavage products; the second lane is substrate and protease in buffer (not formula) to show the basic activity of the protease added to the formula products. The subsequent five lanes show that unmodified formulas cannot affect activity of IgA protease; the Fab fragment is the cleavage product.

Fig. 10B shows that the *Haemophilus influenzae* IgA protease used above in the gel of Fig. 10A is inactive in infant formula (here NUTRAMIGEN®) supplemented by an anti-IgA protease from immune cow colostrum. The bottom two lanes are substrate and enzyme controls. The top four lanes are NUTRAMIGEN® formula containing varying amounts of cow #1 immune colostrum: from top to bottom,

Lactoferrin and non-human antibodies to bacterial
 IgA protease-like proteins or protease precursor-like
 proteins, e.g., bovine IgG capable of inhibiting
 bacterial IgA proteases, can be added to bovine milk-
 based infant formulas. Like human milk, such formulas
 will inhibit IgA protease-like proteins and protease
 precursor-like proteins in the fluids bathing the
 nasopharynx and upper respiratory passages of the infant.
 The advantage of such inhibition is that, in general, the
 child's own IgA will be protected from cleavage by the
 IgA proteases. Thus, all IgA antibodies will benefit,
 not only those directed against the IgA protease
 producing bacteria themselves.

The processed colostrum containing the antibodies
 can be diluted or concentrated and used in combination
 with lactoferrin as an infant formula additive. Standard
 infant formulas include the SIMILAC® line of formulas
 (Ross Laboratories, Columbus, Ohio) and the SMA® line of
 formulas (Wyeth Laboratories, Philadelphia,
 Pennsylvania). Furthermore, the colostrum antibodies
 plus lactoferrin also can be used undiluted as an infant
 formula, with or without added nutrients. The bovine
 antibodies to bacterial IgA proteases can also be
 purified from the colostrum and added along with
 lactoferrin to infant formula directly. For example, the
 antibodies and lactoferrin may be dried into a powder
 form to be added to formulas, or even maternal milk.
 To determine whether the inhibitory effect of the
 bovine antibodies against IgA protease precursor was
 maintained when added to infant formulas, *Haemophilus*
influenzae type 1 protease antibodies were added to
 various infant formulas including SIMILAC®, Enfamil®,
 S.M.A.®, Prosobee®, and NUTRAMIGEN® (Mead-Johnson,
 Evansville, Indiana). Titers were done as for the

FORMULAS AND FORMULATIONS

colostrum titer of 1280 and a milk titer of 20, which were the highest levels of all the cows, even though no inhibition was detectable after the first calving.

Characterization of Bovine IgA Protease Antibody:

5 Using an anti-bovine IgG antiserum we have shown that the IgA protease antibody in bovine colostrum is IgG. This was done by exposing enzymes on western blots to the colostrum preparations as a first antibody, and examining the isotype of the binding antibody by various antibodies 10 against cow serum. Furthermore, chromatography of the inhibiting colostrum on Biogel P200 columns shows that the ability to inhibit co-elutes precisely with the IgG peak.

The inhibiting property of bovine colostrum IgG is 15 localized in the Fab fragment (as anticipated for an antibody). This was shown by using papain to cleave the inhibiting protein into its various fragments as follows. Papain (enzyme/protein ratio of 1/100 w/w) was added to 20 bovine colostrum immunoglobulins and the mixture incubated at 37°C for 16 hours. Proteolysis was stopped with Ca^{++} added in excess, and the material examined on western blot analysis; this showed about 80% hydrolysis. The Fab fragments were isolated by column chromatography 25 on Biogel P100, and identified by their reduced molecular weight (changed elution position) and light chain content as determined by appropriate antisera.

This Fab fragment material was tested as an IgA protease inhibitor, and found to be 2-3 fold less 30 effective than the starting material before proteolysis. Thus, the entire IgG antibody molecule is not required for IgA protease inhibition, although some inhibiting power could be lost by isolation of the monovalent Fab region of the antibody.

correct" assays) are better inhibited than are enzymes from non-matched serogroups. For example, cow #1, immunized with type 1 protease of HI strain Rd, inhibited three out of three serotype d strains (H1Rd, H1D2, and H1D3) with a reciprocal titer of approximately 2,000 (at an IgA protease activity of 50%). IgA proteases from HI of other serotypes (groups b (H1B), e (H1E), f (H1F), and c (H1C)) were inhibited less than 100 at the same level of activity. Fig. 8A shows that enzymes having the same cleavage type as that of the immunogen are better inhibited. Thus, it is preferable to use several enzymes to immunize each cow such that various, e.g., *Haemophilus*, proteases are blocked. Alternatively, antibodies from cows immunized with each protease can be combined for addition to infant formula. Fig. 8B shows the inhibitory curves of cow #236, which was immunized with type 2 IgA protease of HI serotype e. The reciprocal inhibitory titer was 3180. In this case, the three proteases having type 2 specificity (H1C 3 (2), H1E 27 (2), and H1F (2)) were inhibited to a greater extent than the three proteases having type 1 specificity. These curves in Fig. 8B show that in addition to serogroup specificity as shown in Fig. 8A, certain of the bovine antibodies of the invention also show more specificity for enzymes of the correct cleavage type.

Protease-inhibition titers of cows after a second set of immunizations are provided in Fig. 9. Cow #265 had a maximum serum titer at day 21 (titer = 80), but it showed a 4-fold increase in colostrum titer relative to the first lactation (titer = 640). Cow #337 immunized with *N. meningitidis* 2R IgA1 protease had a serum inhibitory titer at day 0 (titer = 40) which increased by day 14 (titer = 80), and reached a maximum at day 21 (titer = 320). After a second calving, this cow had a

activity was expressed as a percentage of the heavy chain that has been cleaved using the formula:

$$\frac{\text{cpm Fd}}{\text{cpm Fd} + \text{cpm heavy chain}} \times 100$$

5 An inhibition assay from the colostrum of 18 cows is shown in Fig. 5. The titers for 18 cows immunized with individual IgA proteases for the first time during a single calving are provided. As is suggested by the figure, the preferred antigens are IgA proteases of *Neisseria* and *Haemophilus*. Inhibiting antibodies were detected in milk obtained after a second immunization and second calving (Fig. 6). The levels of inhibition obtained with colostrum are higher than those obtained with milk, and antibodies from the second calving are 15 more potent than those obtained with the first calving. To determine whether the difference observed between milk and colostrum is due to a difference in the IgG concentrations in the samples, the samples were normalized for IgG level. Even when IgG levels are 20 accounted for, colostrum antibodies were approximately 6 to 10-fold more inhibitory than were milk antibodies. Fig. 7 provides a second set of examples of IgA protease inhibition activities from serum, colostrum, and milk after a first lactation. In this example, 25 inhibiting antibodies were produced when the immunizing antigen was Hie, Hib, *N. meningitidis* 1C or 2R. Generally, antibody titers were greatest in colostrum. Inhibiting antibodies were also obtained in serum at 14 or 21 days after calving (Fig. 7). Experiments also show that the bovine colostrum 30 antibodies of the invention differ in inhibiting titre against various IgA proteases. Fig. 8A shows that enzymes from strains sharing the same serogroup with that of the source of the immunizing enzyme (i.e., "serotype

Generally, the assay involves quantitating the amount of IgA1 Fab produced over time. The Fab produced can be measured by measuring the heavy chain of the Fab region. A typical reaction mixture has a volume of 25 μ l consisting of 2% labeled IgA1, 25 μ l of a Tris/HCl/BSA buffer containing the substrate solution (2 mg/ml purified IgA1 with 2% labeled IgA1), 25 μ l of a colostrum dilution control, and 25 μ l of active IgA protease (Haemophilus, Neisseria or streptococcal species, but any were incubated for 30 minutes at 37°C to allow them to interact, the same substrate was added, and incubation continued at the same temperature. The aliquots of 10 μ l were removed at 10-minute intervals over a 40 minute period and hydrolysis of IgA1 measured as follows. The aliquot was added to 100 μ l of sample buffer for SDS-PAGE, and boiled 5 minutes. The sample buffer contains 1.2% mercaptoethanol, and 0.001% sodium dodecyl sulfate, 1.2% glycerol, and 0.05% Coomassie Brilliant Blue, dried, and autoradiographed at -70°C. Aliquots of 100 μ l were stained with 0.05% Coomassie Brilliant Blue, dried, and autoradiographed at -70°C. The 25 using developed the stained and dried gel into segments for cutting of 1) any uncleaved heavy chain and 2) the 22 kD α chain within the uncleaved heavy chain and in its product were counted on a Beckman Biogamma Counter. Radioactivity in the uncleaved heavy chain and in its product were counted from control digests of IgA protease from each value, and quantitation of IgA protease

added to infant formulas in the same way as the bovine antibodies or bovine antibody containing colostrum described above.

Monoclonal antibodies useful in the invention can be made by standard techniques by immunizing mice with all or a portion of an IgA protease precursor-like

protein, fusing the murine splenocytes with appropriate myeloma cells, and screening the antibodies produced by the resultant hybridoma lines for the requisite ability to bind the IgA protease precursor-like protein (e.g., by means of an ELISA assay). A subsequent screening may be necessary to select binding antibodies that also inhibit or remove the protease or protease precursor. Monoclonal antibody production and screening can be performed

15 according to Uchiyama et al. (*J. Immunol.* 126:1393, 1981). Alternatively, useful antibodies may be isolated from a combinatorial library produced by the method of Huse et al. (*Science* 246:1275, 1989).

The invention can employ not only intact polyclonal or monoclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or (Fab)₂ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778); or a chimeric antibody, for example, an antibody that contains the binding specificity of a murine or bovine antibody, but in which the remaining portions are of human origin.

IgA Protease Inhibition Assay: A suitable enzyme

30 assay uses human myeloma IgA1 immunoglobulin trace labelled with ¹²⁵I as a substrate. Fragmentation products of the IgA1 heavy chain are measured by first separating these fragments on polyacrylamide gels. A suitable antibody inhibits protease activity by binding to the protease precursor or the protease.

adjuvant Quil A. Each immunization employed 3 ml saline and 1 mg IgA protease (or 6 mg IgA protease for a "cocktail"). Injections were given approximately 28 and 14 days before expected calving. Inoculation was in four sites: 1 ml injected subcutaneously into each shoulder, and 0.5 ml injected intramuscularly into the gluteal region on each side). The maternal colostrum (approximately four liters) then was collected by venipuncture (bovine colostrum whey proteins typically consist of 40% immunoglobulins, 35% beta-lactoglobulin, 15% lactalbumin and small amounts of albumin and other proteins). The colostrum was centrifuged for 30 minutes at 12,000 x g at 4°C to remove the fat (which floats to the top of the sample). The casein then was precipitated by incubating the colostrum for 1 hour with 15 units of rennin. The curd subsequently was tightened by heating the sample to 56°C for 10 minutes, and the casein was removed by centrifugation at 4°C for 25 minutes at 12,000 x g. If desired, the colostrum may be frozen at this point.

To produce the formulas and formulations of the invention, active lactoferrin is added to the processed colostrum or milk to a concentration of 0.01-10 mg/ml, preferably 0.5-1.0 mg/ml. After lactoferrin is added, the pH of the formula should not be permitted to drop below pH 4.0, preferably pH 5.0, and the formula should not be heated significantly (e.g., 90°C for 5 minutes, or 56°C for 30 minutes). Generally, lactoferrin is active at a temperature 18-37°C.

The colostrum or milk may be tested by culture for sterility, and it can be frozen until used.

Monoclonal IgA Protease Antibodies: Monoclonal antibodies directed against bacterial IgA protease precursor-like proteins also can be used according to the invention. These antibodies or antibody fragments can be

Method of Producing Antibodies That Specifically Bind IgA Protease- or Protease Precursor-like Proteins:

To produce the infant formulas, infant formula additives, or pharmaceutical formulations of the invention, a non-human mammal, e.g., a cow, is immunized with a bacterial IgA protease precursor or protease precursor-like protein or a peptide fragment thereof (e.g., the mature IgA protease). In general, bovine immunization to obtain milk, or preferably colostrum, containing antibodies is preferably performed as follows. During the last month of gestation, the cow transfers a very large amount of circulating plasma IgG into its udder. A parenteral (subcutaneous, intramuscular, etc.) injection of an IgA protease (or the preferred antigen) is preferably made during this period to stimulate high levels of circulating (plasma) antibody that eventually are transferred to the colostrum and milk.

This colostrum is then processed by conventional methods to produce the infant formula, formula additive, or pharmaceutical formulation. Because the desired activity of lactoferrin is sensitive to heat and acidification, processing steps which involve heat or acidification should not be carried out after lactoferrin is added. When a person (e.g., an infant) drinks the formula containing the antibody and lactoferrin, the formula protects the mucosa in all of the areas where ingested milk normally contacts the mucosa, i.e., the oral cavity, nasopharynx, bronchial passages, and intestine. Because simple ingestion of maternal milk normally is sufficient to deliver antibodies to these mucosal surfaces, ingestion of the formula of the invention is expected to provide an adequate means for delivery of the formula to the preferred mucosa.

In one example, pregnant cows received two injections of IgA proteases mixed with 1 mg of the

Combined Effect of Lactoferrin and Antibodies
which Bind IgA Protease- or Protease Precursor-Like
Proteins: The invention provides infant formulas which
 5 inhibit bacterial IgA proteases from cleaving human IgA,
 the only known substrate for this class of proteases.
 Formulations that include lactoferrin and antibodies that
 inhibit IgA proteases or IgA protease precursors or
 10 related proteins also can be used to inhibit the
 pathogenic effects of bacteria expressing these proteins
 in adults.
 As is reported above, lactoferrin extracts IgA
 proteases or protease precursor-like proteins from the
 15 outer membranes of pathogenic bacteria. Once extracted,
 the proteases or protease precursor-like proteins are
 targeted by antibodies provided in the formula or
 formulation. Useful antibodies are those which
 specifically bind the protease precursor or protease
 20 precursor-like protein or a processed protease or
 protease-like protein. Antibodies that bind the
 precursor-like protein prevent processing of the
 precursor-like protein, while antibodies that bind the
 protease-like protein directly inhibit the activity of
 25 the protease. Accordingly, the antibodies which are used
 in the invention can be raised against all or a portion
 of the mature IgA protease or protease-like protein, or
 all or a portion of the IgA protease precursor or
 protease precursor-like protein. Many, if not all,
 30 antibodies raised against such proteins will be able to
 inhibit the ability of the protease precursor to process
 itself, or the ability of the protease to cleave its
 target molecule (e.g., IgA). Methods for preparing
 appropriate antibodies are described below.

agglutinin, facilitating attachment of chicken *E. coli* to epithelial cells. Thus, extraction of this protein with lactoferrin alone, or in combination with antibodies directed against Tsh (e.g., antibodies raised to the extracted and gel-purified protein), is a means for inhibiting septicemia caused by this pathogen in animals. Also included is the HAP protein of HI. This protein is associated with the ability of the organism to bind and enter host epithelial cells. The protein shares sequence identity with the catalytic domain of IgA protease and with Tsh protein. The HAP outer membrane protein is processed in a manner similar to that of IgA protease. Extraction of any of these proteins, combined with binding of the protein with antibodies (e.g., antibodies raised against the extracted and gel-purified protein), is expected to inhibit the pathogenic effects of these bacteria (e.g., by precipitating the HAP protein).

Specificity of the Lactoferrin Effect: We next examined whether lactoferrin specifically extracts IgA protease precursor-like proteins from the outer membranes of cells, or if other cell surface proteins also are extracted. To this end, proteins on HI cells were labeled with ³⁵S-methionine, and the cells were treated with lactoferrin. The IgA protease precursor constituted approximately 80% of the labeled protein that was extracted from HI. When a similar labeling experiment was performed with an *iga*⁻ mutant of HI which does not express the IgA protease, virtually no extracted proteins were detected. These data suggest that, in HI, the effects of lactoferrin are specific for IgA protease precursor-like proteins.

Streptococcus sanguis. The *S. sanguis* IgA protease lacks substantial homology to HI IgA protease, and differs from that of HI in that it is a metalloprotease rather than a serine protease. After 6-8 hours of growth, 50% of the IgA protease of this bacterium stays on the cell, and 50% is released into the culture supernatant. The membrane-bound protease is thought to play a role in initiation of attachment and colonization of other pathogenic bacteria on the surface of saliva-coated teeth. We have discovered that the addition of lactoferrin to a culture of *S. sanguis* causes approximately 70% of the IgA protease to be released in 1 hour; by contrast, only 10% of the protease is released when cells are grown for 1 hour in the absence of lactoferrin. These data thus indicate that the lactoferrin effect is not limited to gram negative bacteria, as lactoferrin is able to remove the protease from *S. sanguis*. Once extracted, the activity of the protease can be inhibited with antibodies (e.g., antibodies raised against the extracted and gel-purified protease) according to the invention.

IgA Precursor-like Proteins: Proteins from several other bacteria also are functionally or structurally related to the HI IgA protease precursor, and lactoferrin is expected to extract these IgA protease precursor-like proteins from cell membranes. For example, the precursor-like protein of vacuolating cytotoxin of *Helicobacter pylori* has a helper section, and the protein is substantially similar to IgA protease. Extraction and neutralization of this toxin can protect against conditions, such as chronic gastritis, which are attributed to *H. pylori*. Similarly, the Tsh protein of chicken *E. coli* shares over 60% sequence identity with HI IgA protease precursor. Like the IgA protease precursors, the Tsh protein includes a helper section. Tsh serves as an

western blot analysis using antibodies directed against the IgA protease. These results indicate that the IgA protease precursor remains substantially unprocessed in milk containing signal.

5 In contrast, the protease precursor is processed over time when it is extracted into milk that lacks signal (i.e., jacalin-treated milk) (Fig. 4B). The addition of signal to signal depleted milk blocks processing of the protease precursor. Additional experiments indicate that 10 bovine IgG obtained from milk of a cow immunized with the IgA protease can replace the signal of human milk and block processing of the protease precursor. In sum, these data indicate that lactoferrin can be used in conjunction with antibodies to extract the protease precursor from the 15 cell membrane and inhibit its processing in the mature enzyme.

Additional data indicate that the combination of lactoferrin and antibodies directed against the IgA protease is sufficient to inhibit the ability of the 20 extracted IgA protease to cleave IgA. Here, HI cells were grown to mid-log phase, washed with saline, then aliquotted into 0.3 ml cultures. The cultures were incubated for 1 hour in 0.2 ml of milk, IgA depleted milk, or IgA depleted milk supplemented with IgA. The 25 cells were pelleted and washed with saline. The IgA protease activity in 30 μ l aliquots of the supernatants was detected by assaying the supernatants for cleavage of radioactively labeled IgA. As is illustrated in Fig. 4C, the combination of lactoferrin and IgA antibodies 30 blocks the IgA protease from cleaving IgA.

Use of Lactoferrin to Extract IgA Protease From a Gram Positive Bacterium: To determine whether lactoferrin also is able to extract the membrane-bound IgA protease from gram positive bacteria, we measured the 35 ability of lactoferrin to extract the IgA protease of

dominant factor responsible for extraction of the protease precursor.

The ability of lactoferrin to extract the IgA protease precursor from a cell membrane is sensitive to heat, pH, and the presence of chelators such as EGTA. Extraction of the IgA protease precursor was not detected when the whey was treated at 56°C for 30 minutes or 90°C for 5 minutes prior to incubating the whey with the HI cells. These data indicate that lactoferrin activity is lost by heating the protein (e.g., by pasteurizing milk). Similarly, the lactoferrin activity is inhibited by treating the lactoferrin with EGTA. In addition, adjusting the pH of the lactoferrin to a pH of 4.0 or below for 10 minutes, followed by neutralization of the pH, abolishes the ability of the lactoferrin to extract the IgA protease precursor. Accordingly, we have found that bovine milk that has been pasteurized or acidified (e.g., in removing casein) lacks this desired activity of lactoferrin. In addition, iron resaturation of acidified lactoferrin does not restore this activity of lactoferrin. However, fresh bovine milk is able to extract the IgA protease precursor from the outer membrane.

Processing of the Extracted Protease Precursor:

Processing of the extracted protease precursor into the mature protease is inhibited by secretory IgA1 (sigA1). This result is illustrated in Figs. 4A-C. In this experiment, HI cells were grown to mid-log phase in BHI, and 0.6 ml aliquots were incubated at 37°C for 45 minutes in milk, sigA1-depleted milk, or sigA1-depleted milk supplemented with sigA1. Following the incubation in milk, the cultures were centrifuged, and the cell-free milk supernatants were further incubated at 37°C for various lengths of time (0.75 to 24 hours) (Figs. 4A-C). The IgA protease precursor subsequently was detected by

membrane of HI. The ability of human lactoferrin (Sigma cat. No. L-0520) to extract the IgA protease precursor is illustrated in Fig. 2. In this example, IgA mutant HI cells were grown to mid-log phase in BHI medium, then incubated in human lactoferrin at a concentration of 4 mg/ml or 2 mg/ml. Like whey, substantially purified human lactoferrin was able to extract the protease precursor from the cell membrane. In contrast, incubation of the cells in TBS buffer did not result in release of the protease precursor from the cell membrane. Recombinant lactoferrin was used to confirm that lactoferrin extracts the IgA protease precursor from cells. This recombinant lactoferrin was produced in eukaryotic cells, and is iron-saturated. In this experiment, HI IgA mutant cells were grown to mid-log phase in BHI, then incubated for 1 hour in 0.5 mg/ml recombinant lactoferrin. As illustrated in Fig. 3, recombinant lactoferrin also extracted the entire IgA protease precursor from the outer membranes of wild type (HI Rd⁻) and two mutant strains (HI 3-13 and HI 2-25DK) of HI. Interestingly, the IgA protease precursor extracted with lactoferrin from each of the above sources, including recombinant lactoferrin, also exhibited a decreased mobility by SDS-PAGE, providing further evidence that a milk-derived adduct is not responsible for the apparent molecular weight. Human plasma containing serum transferrin and purified serum transferrin were also tested for their ability to extract the IgA protease precursor from cell membranes. Serum transferrin is approximately 20% as effective as lactoferrin in extracting the IgA protease precursor from the membrane, suggesting that the iron-transporting property of these proteins is not the

35 extract the IgA protease precursor from the outer
active, human and bovine lactoferrin each were able to
commercially available substantially purified, but
its ability to extract the IgA protease precursor.
30 Agent: lactoferrin from several sources was assayed for
To confirm that lactoferrin is the extracting
Confirmation that lactoferrin is the extracting
which causes extraction of the IgA protease precursor.
analysis was used to identify lactoferrin as the agent
25 gel filtration column. From the final pooled column
flowthrough then separated by size on a BioGel P-200
column, with the desired activity, western blot
the whey was fractionated on a DE-52 anion exchange
20 classical protein purification methods were used. First,
To isolate the extracting agent from whey, first,
precipitate the IgA protease precursor from whey, first,
15 acetone to precipitate the extracting agent from whey, first,
apparent molecular weight. To determine whether the
lipids are able to modify cell membranes, and they have been shown to
10 antimicrobial properties, and they have been shown to
precursor from cell membranes. In addition, milk
factor responsible for extraction of the IgA protease
lines of reasoning suggested that a milk lipid was the
5 extracted 240 kD protein is a degradation product of the
protease precursor. These and other data also suggest
extracted species match the N-terminus of both the 190 kD and 240 kD
confirms that the N-termini of both the 190 kD and 240 kD

was detectable in an extraction reaction that included ¹²⁵I-labeled milk proteins.

In contrast, these same antibodies specifically bound the IgA protease, the IgA protease precursor, and a recombinant helper section of the IgA protease precursor. In addition, these antibodies crossreact with the 210 kD unprocessed IgA protease precursor of an HI strain which has a mutation in the *iga* gene. These results indicate that the protein which migrates as a 240 kD species is the extracted IgA protease precursor. The data also indicate that the helper section is responsible for the apparent shift in the molecular weight of the IgA protease precursor.

When cells are grown in a conventional culture medium in the absence of lactoferrin, the enzyme portion of the IgA protease is no longer attached to the membrane; however, the helper section remains in the membrane. Treatment of cells having a membrane-bound helper section with whey leads to extraction of the helper section of the IgA protease precursor. Treatment of cells having an intact membrane-bound protease precursor with whey leads to extraction of the unprocessed protease precursor. Like the full-length extracted IgA protease precursor, the extracted helper section exhibits a decrease in mobility by SDS-PAGE. Taken together, these data lead to the conclusion that growth of HI in whey leads to extraction of the entire IgA protease precursor or the helper section of the protease precursor from the outer membrane of the cell. Although most of the extracted protein migrates with a decreased mobility by SDS-PAGE, the antibody crossreactivity of the extracted species indicates that the extracted protein having an apparent molecular weight of 240 kD is the extracted IgA protease precursor. In addition, N-terminal sequencing of the extracted proteins

In addition, the ability of whey to extract the IgA
 protease precursor from the cell is proportional to the
 concentration of whey in the sample. Dilution of the
 whey led to a diminished ability to extract the IgA
 protease precursor from the cell. In contrast to the
 behavior of the IgA protease in milk, the protease was
 not extracted when cells were grown for 80 minutes in
 saline (Figs. 1A and 1B, lane 8). These data thus
 indicate that a component of milk promotes extraction of
 the entire IgA protease precursor from the cell.

10 In this experiment, the predominant protein
 species detected with anti-IgA protease antibodies
 migrated through an SDS-polyacrylamide gel as though it
 were approximately 240 kD in size. A less prevalent
 15 species also was present, and it migrated as though it
 were 190 kD in size, the predicted molecular weight of
 the IgA protease precursor. Several lines of evidence
 indicate that the species migrating as 240 kD and 190 kD
 both are related to the protease precursor.

20 To determine whether the apparent shift in the
 molecular weight was due to glycosylation, the protein
 was treated with glycosidases. The negative results in
 these experiments suggest that glycosylation is not
 25 likely to be the cause of the apparent shift in the
 mobility of the protein. We also examined the
 possibility that the apparent molecular weight is caused
 by an adduct from the milk in which the HI cells were
 grown. To test this possibility, antibodies were raised
 30 against the extracted 240 kD protein. These antibodies
 then were used to probe a sample of milk. However,
 nothing in milk crossreacted with the antibodies,
 suggesting that milk does not contain an adduct
 responsible for the apparent molecular weight of the
 extracted IgA protease precursor. In addition, no adduct

precursor can be further processed to release the distinct from the processing site of the membrane-bound protease precursor (the A site). In the presence of inhibitory antibodies, processing of the extracted protease precursor at the C site is blocked. Experiments demonstrating this phenomenon are described below.

Extraction of IgA Protease Precursor in Whey:

This experiment demonstrates that the IgA protease precursor of HI undergoes alternative processing when a normal milk whey (i.e., de-fatted milk) is used as the culture medium. This experiment employed a strain of *Haemophilus influenza* (HI) that has a mutation in the *iga* gene encoding the IgA protease precursor. The mutant IgA protease precursor is unable to cleave the mature protease off of the protease precursor.

In this experiment, the mutant HI cells were grown in a BHI medium until they reached mid-log phase (approximately 5 hours). Several 0.4 ml aliquots of cells were taken from the culture, and the cells were washed once with saline. The 0.4 ml aliquots of cells then were grown in 0.2 ml whey for various lengths of time (2, 5, 10, 20, 40, or 80 minutes). As a control, an aliquot of cells was grown in saline for 80 minutes. At the ends of the incubation periods, the cells were harvested by centrifugation, and 25% of the cell pellet and 100 μ l of the milk supernatant from each culture were analyzed by western blot analysis using antibodies raised against gel-purified the IgA protease.

Growth of HI cells in milk results in extraction of the IgA protease precursor from the cell surface into the culture supernatant. As is illustrated in Figs. 1A and 1B, over time, the IgA protease precursor becomes undetectable in the cell pellet, and it appears in the culture supernatant (Figs. 1A and 1B; compare lanes 2-7).

Fig. 10A is a reproduction of an autoradiogram of an electrophoresis gel showing the activity of IgA protease when added to infant formulas.

Fig. 10B is a reproduction of an autoradiogram of an electrophoresis gel showing the inactivity of IgA protease when added to infant formulas supplemented with the bovine antibodies of the invention.

PROCESSING OF IgA PROTEASE PRECURSOR AND PRECURSOR-LIKE PROTEINS

Normal Processing of an IgA Protease Precursor-Like Protein: In conventional growth media, IgA protease precursor-like proteins are proteolytically processed into the mature enzymes in reactions that may be autocatalytic in nature. For example, the IgA protease precursor of HI exists as a 184 kD transmembrane protein that undergoes autocatalytic cleavage to produce a 109 kD secreted enzyme. The 70-80 kD helper section (i.e., beta domain) remains docked in the outer membrane of the cell. On an SDS polyacrylamide gel, the observed sizes of these proteins is larger than the predicted sizes. The IgA protease precursor migrates as a species of 210 kD; the secreted enzyme migrates as a species of 116 kD; and the helper section migrates as a species of 120 kD.

Alternative Processing of an IgA Protease Precursor-Like Protein: Applicants have discovered that, when bacteria expressing a cell-surface IgA protease precursor-like protein are contacted with lactoferrin, the IgA protease precursor-like protein undergoes an alternative form of processing. For example, when an HI cell containing an IgA protease precursor is treated with lactoferrin, the entire protease precursor is extracted from the cell. Extraction of the entire protease precursor occurs at only low levels (10-20%) in normal growth media or buffer lacking lactoferrin. In the absence of inhibitory antibodies, the extracted protease

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Detailed Description

5 The drawings are first briefly described.

Drawings

Figs. 1A and 1B are photographs of gels showing that the IgA protease precursor is extracted when the cells are grown in whey.

10 Fig. 2 is a photograph of a gel showing that the IgA protease precursor is extracted when the cells are grown in substantially pure human lactoferrin.

Fig. 3 is a photograph of a gel showing that the IgA protease precursor is extracted when the cells are grown in pure, recombinant lactoferrin.

Figs. 4A to 4C are photographs of gels showing that processing of the extracted IgA protease precursor is inhibited in the presence of secretory IgA1 (sIgA1).

20 Fig. 5 is a chart showing the titers obtained after immunization of 18 cows with IgA proteases.

Fig. 6 is a chart showing the titers obtained after a second immunization and calving.

Fig. 7 is a chart providing a second example of titers obtained after immunization of cows with IgA protease.

25 Figs. 8A and 8B are graphs showing the difference in inhibiting titre against various IgA proteases having the same or different serogroups as the immunizing enzyme.

30 Fig. 9 is a chart providing the protease inhibition titers of cows after a second set of immunizations.

species (e.g., a goat) from which milk or colostrum of the formula is derived.

The invention offers several advantages. By using lactoferrin to extract the IgA protease precursor-like protein from the cell membrane, and by using antibodies directed against the protease precursor-like protein or mature protease-like protein, the invention provides a means for inhibiting every IgA protease or protease precursor-like protein in a sample. By so doing, every IgA1 antibody of every specificity in the person (e.g., infant) will be available to contribute to a protective immune response. This type of protection against the bacterial IgA protease does not require killing the bacterium. In addition, immune complexes formed by antibodies that bind the IgA protease or its precursor can be delivered to M cells for generation of an immune response more readily than is the IgA protease or protease precursor in the absence of such antibodies. Applicants have also found that IgA protease precursors from gram negative cells and proteases from gram positive cells are extracted by lactoferrin and thus are both susceptible to neutralizing antibodies. This effect of lactoferrin is unaffected by iron-saturation of the lactoferrin, thus expanding the applicability of the method.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

precursor-like protein, and which inhibit the proteolytic activity of the protein.

As used herein, the term "protease" includes not only the complete protease but immunologically-active

5 fragments thereof, which include any peptide fragments that give rise to antibodies, e.g., against the complete IgA protease, when used as an immunogen.

By "active" lactoferrin is meant lactoferrin which

10 retains the ability to extract an IgA protease precursor or protease precursor-like protein from the membrane of a cell. Generally, lactoferrin is active if it is purified or produced without substantial heating (substantial

heating is considered incubation at 56°C for 30 minutes or 90°C for 5 minutes) or acidification (incubation at pH 15 4.0 or below). Generally, conventional pasteurization techniques (e.g., heating milk for 30 minutes at 68°C) inactivate lactoferrin. Generally, lactoferrin is

inactive in the presence of 15 mM EGTA or EDTA. As used herein, the term active lactoferrin includes derivatives of, and those portions of lactoferrin that retain the

20 above-described extraction activity. The lactoferricin domain of lactoferrin is not in and of itself considered active lactoferrin because it is insufficient to extract the protease precursor. Active lactoferrin can be

25 produced with recombinant DNA techniques (e.g.,

purification from a eukaryotic cell engineered to express lactoferrin *in vitro*). Alternatively, the exogenous

lactoferrin can be purified from a natural source of lactoferrin. For example, purified active bovine

30 lactoferrin can be admixed with pasteurized bovine colostrum or milk containing appropriate antibodies in

preparing an infant formula of the invention.

Alternatively, the active lactoferrin may be derived from a species (e.g., a cow) which is different from the

used in the invention since there is no apparent requirement for the carbohydrates found in naturally occurring lactoferrin. In addition, conventional gene expression methods can be used to overexpress lactoferrin (e.g., in yeast, baculovirus, or in a mammal (e.g., a transgenic cow)), and lactoferrin obtained from such a mammal can be used in the invention.

If desired, colostrum or milk used in the invention can be processed before preparing the infant formulas of the invention. For example, fat and casein may be removed. As is discussed herein, processing steps which include heating or acidification should not be performed after addition of lactoferrin to a formula. If desired, processing can include the step of reducing the volume of the colostrum by filtering through a 100,000 MW filter. In addition, antibodies (e.g., IgG) can be purified from milk, combined with lactoferrin, and used as a formula additive.

As used herein, the term "infant formula" includes colostrum or other milk preparations that include the inhibitors or antibodies of the invention and, if desired, purified inhibitors that are fed to an infant orally in combination with any other suitable beverage. The term "antibody" includes not only complete antibodies but immunologically-active fragments thereof, which include any antibody fragments that are effective to inhibit the proteolytic activity of IgA proteases, IgA protease-like proteins, IgA protease precursors, or IgA protease precursor-like proteins. Antibodies raised against the entire protease precursor-like protein, the helper section of the precursor-like protein, or the mature protease-like protein all are included. Also included are monoclonal antibodies that specifically bind any portion of the IgA protease-like protein or

protease precursor and that does not specifically bind an IgA protease. For example, an antibody that specifically binds the helper section of an IgA protease precursor is included within the invention.

In preferred embodiments, the infant formula includes non-human milk or colostrum, such as cow's colostrum. As used herein, the term "milk" encompasses colostrum and the subsequent lacteal secretions.

Preferably, the formula includes colostrum (i.e., the antibody-rich first secretion at the termination of pregnancy).

In a preferred embodiment, the immunization is performed during the last month of gestation, and the antibodies are collected by collecting colostrum.

Preferably, the animal (e.g., cow) is immunized during each of a first and a second pregnancy, and the colostrum or milk is collected after the termination of the second pregnancy since colostrum or milk collected at that point has higher levels of the desired antibodies. Preferably, the antibody which specifically binds an IgA protease precursor-like protein binds a vacuolating cytotoxin

(e.g., from *Helicobacter pylori*), a Tsh protein (e.g., chicken *E. coli* Tsh protein), or a hemagglutinating adherence protein (HAP protein).

The lactoferrin which is employed in any of the various aspects of the invention can be derived from any mammal; preferably, the lactoferrin is human or bovine in origin. Lactoferrin from other ruminants (e.g., sheep and goats) is also preferred. Such lactoferrin can be isolated from a secretion (e.g., milk) of the mammal, or the lactoferrin can be produced with recombinant DNA techniques using standard techniques. Recombinant lactoferrin used in the invention can be produced in vitro or in vivo. For example, lactoferrin produced in cultured mammalian tissue is suitable in the invention.

Lactoferrin produced in a prokaryotic cell can also be

active (e.g., non-pasteurized) lactoferrin to produce the infant formula additive.

The invention further features a method for inhibiting processing of an IgA protease precursor on a cell. This method involves contacting the cell with a formulation comprising a pasteurized excipient, and active (e.g., pasteurized) lactoferrin, thereby producing an extracted IgA protease precursor, and contacting the extracted IgA protease precursor protein with an antibody which specifically binds (i.e., inhibits the proteolytic activity of) the IgA protease precursor.

In yet another aspect, the invention features a method for inhibiting proteolysis of an IgA1 antibody by an IgA protease. This method involves contacting a cell which includes a precursor of the IgA protease with a formulation that includes a pasteurized excipient, and active (non-pasteurized) lactoferrin, thereby producing an extracted IgA protease precursor. The method also involves contacting the extracted IgA protease precursor with an antibody which specifically binds (i.e., inhibits) the proteolytic activity of the IgA protease. Also within the invention is a pharmaceutical formulation which includes a pasteurized excipient;

active (e.g., pasteurized) lactoferrin; and an antibody that binds (i.e., inhibits) the proteolytic activity of an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor. Similarly, the invention includes a pharmaceutical formulation comprising active (e.g., pasteurized) lactoferrin and an antibody that specifically binds (i.e., inhibits) an IgA protease-like protein, an IgA protease precursor-like protein, or both an IgA protease-like protein and an IgA protease precursor-like protein. Finally, the invention features a monoclonal or polyclonal antibody that specifically binds an IgA

Applicants have discovered that, in the presence of lactoferrin, IgA protease precursor undergoes an alternative form of processing, which renders the entire IgA protease precursor susceptible to neutralizing antibodies which specifically bind the precursor. Accordingly, the invention features, in one aspect, an infant formula which includes pasteurized milk (e.g., colostrum), active lactoferrin (e.g., non-pasteurized lactoferrin), and an antibody which specifically binds (i.e., inhibits the proteolytic activity of) either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor. In a second aspect, the invention features a method for preparing a pasteurized infant formula. The method involves immunizing a non-human mammal with all or a portion of an IgA protease or an IgA protease precursor; collecting from the mammal milk (e.g., colostrum) including antibodies which specifically bind (i.e., inhibit the proteolytic activity of) an IgA protease or an IgA protease precursor, or both; pasteurizing the milk; and preparing the infant formula using the pasteurized milk and active (e.g., non-pasteurized) lactoferrin. The invention also features a method for producing an infant formula additive, involving immunizing a pregnant non-human mammal with all or a portion of an IgA protease or an IgA protease precursor; collecting from the mammal milk (e.g., colostrum) including antibodies which specifically bind (i.e., inhibit the proteolytic activity of) an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor; pasteurizing the milk; and mixing pasteurized milk with

Summary of the Invention

children. Conditions caused by these bacteria include
 otitis media, pneumonia, bronchitis, sinusitis,
 septicemia and meningitis. These bacterial infections
 are a major problem in all countries and even in major
 U.S. cities. In the United States, for example, 67% of
 all children develop otitis media by the age of three
 years (Mandell et al., Principles and Practice of
 Infectious Disease (3d ed.), p. 506, Churchill
 Livingstone, N.Y. 1990). HI is the predominant pathogen
 10 that causes otitis media.

IgA proteases are encoded as a single large
 polypeptide chain by *iga* genes in the bacterial
 chromosome. The proteases are secreted from bacterial
 cells by an autocatalytic processing mechanism.
 15 Bacterial IgA proteases cleave and inactivate human IgA
 on mucosal surfaces and, in this way, allow bacterial
 pathogens to evade the mucosal immune mechanisms. IgA
 cleavage impairs function by dissociating the Fab and Fc
 regions of IgA. In addition, free Fab fragments may also
 20 bind key microbial antigens, blocking access by other
 protective antibodies.

Certain infant formulas have been designed to
 protect the infants that drink the formula against
 25 bacterial and rotavirus infections. Modification of
 infant formula typically involves immunizing a pregnant
 cow with certain bacteria or their toxins, and then
 extracting the desired antibody from the colostrum (the
 first milk after calving) for addition to formula
 products. Such antibodies are known to protect children
 30 against infection by several intestinal pathogens
 including enterotoxigenic *E. coli*, *Vibrio cholerae*, and
 rotaviruses.

INFANT FORMULA AND INFANT FORMULA ADDITIVES

Background of the Invention

This invention relates to infant formulas and infant formula additives that help protect against infection.

Adults have high concentrations of IgA in their

secretions, and this immunoglobulin isotype defends

against infection at mucosal surfaces. In contrast, the normal newborn child has no mucosal IgA of its own. To

make up for this deficiency, human milk is rich in IgA

(averaging about 120 mgm/dl, but varying widely through

the months of lactation), and provides passive immunity

by bathing the infant mucous membranes, e.g., the oral

cavity, pharynx, bronchi, and intestine with antibodies.

Human milk IgA antibodies have specificity reflecting the antigen experience of the mother, and those antigens to

which the child is also likely to be exposed. During the first six months of life, however, IgA slowly appears in

the child, carrying antibody specificity for bacteria,

viruses, food proteins and other mucosal antigens.

Therefore, the maternal/infant collaboration consists of initial "passive" antibodies from the mother's milk

during the period in which the child's secretory immune system matures, and then the child's own "active"

antibodies.

Bacterial colonization involves the attachment and proliferation of bacteria to mucosal surfaces. Under

certain circumstances these microorganisms cause disease. certain bacteria that colonize and infect infants

continuously secrete IgA proteases that can cleave both maternal and infant IgA. For example, all strains of

Haemophilus influenzae (HI), *Neisseria meningitidis*, and *Streptococcus pneumoniae* that have been studied produce

these IgA proteases and cause serious infections in

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(54) Title: INFANT FORMULA AND INFANT FORMULA ADDITIVES
(57) Abstract

Disclosed are infant formulas, infant formula additives, and pharmaceutical formulations that include active lactoferrin combined with antibodies which specifically bind IgA protease-like proteins or IgA protease precursor-like proteins. The formulas protect persons who drink the formulas or formulations from the pathogenic effects of bacteria and other infectious agents, e.g., viruses, which infect the upper respiratory tract.